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Review

Transport of proteins across or into the mitochondrial outer membrane

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ABSTRACT

Mitochondria are surrounded by two biological membranes. The outer mitochondrial membrane contains two major translocators, the TOM40 (TOM) and TOB/SAM complexes for protein translocation across and/or insertion into the outer membrane. The TOM40 complex functions as an entry gate for most mitochondrial proteins, and the TOB/SAM complex as a specialized insertion machinery for β-barrel membrane proteins. In order to handle loosely folded or unfolded precursor polypeptides, those translocators cooperate with chaperones in the cytosol and intermembrane space, and also exhibit chaperone-like functions on their own. Several α-helical membrane proteins take 'non-standard' routes to be inserted into the outer membrane. Here we review the current view on a remarkable variety of mechanisms of protein transport taking place at the mitochondrial outer membrane.

1. Introduction

Mitochondria are bounded by two membranes, the outer and inner membranes, which separate two aqueous compartments, the intermembrane space (IMS) and innermost matrix. Reflecting the prokaryotic origin, mitochondria have their own genome and protein synthesis systems. However, extensive transfer of mitochondrial genes to the nuclear genome during evolution has resulted in only a minimal set of genes retained in the mitochondrial DNA, yet vast majority of mitochondrial proteins being translated in the cytosol. Cytosolically translated mitochondrial proteins are imported back into mitochondria and sorted to one of the four mitochondrial subcompartments.

Mitochondria contain about 1000–1500 different proteins. Import and subsequent intramitochondrial sorting of mitochondrial proteins are mediated by membrane protein complexes called translocators (often called translocons or translocases as well) in the outer and inner membranes and soluble factors in the cytosol, IMS, and matrix. The TOM40 complex (also called the TOM complex) in the outer membrane and the TIM23 and TIM22 complexes in the inner membrane had been regarded as major protein translocators in mitochondria until 2003.[1] However in these 6 years, new translocators and new components mediating mitochondrial protein transport, e.g. the TOB/SAM complex in the

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[1] In 1996, 10 research groups in the field of mitochondrial protein import agreed to establish a new TOM/TIM nomenclature that could unify the divergent names for components of membrane-linked protein transport machineries of mitochondria in several different organisms [1]. At the time, only one transport machinery was found in each of the mitochondrial membranes, so that they were called the TOM and TIM complexes. When the second transport machinery was identified in the mitochondrial inner membrane, it was named the TIM22 complex whereas the former TIM complex was renamed to the TIM23 complex; the numbers represent the central or channel component of each machinery (Tim22 and Tim23). Therefore if a second transport machinery was identified in the outer mitochondrial membrane, the TOM complex would be renamed to the TOM40 complex (Tom40 is the central channel component) while the new machinery would be named the TOMxx complex (xx stands for the molecular weight in kDa of the central channel component of the new machinery). However for some reasons, when a new transport machinery for β-barrel protein assembly was identified in the outer membrane in 2003, it was named the TOB complex and SAM complex by two different groups. In parallel with this, several newly identified components did not follow the original unified TOM/TIM nomenclature, but instead received names such as Pam, Mia, and Mim. On the other hand, since new components were identified mainly in yeast, the standard nomenclature in the yeast researchers community, the Saccharomyces Genome Database (SGD), may have alternative legitimacy for naming of the components, although it is based on the rule of 'first comes, first served' and lacks agreement among research groups in the field of mitochondrial protein import. Therefore at the moment, it is too early to decide which is more appropriate, the unified and agreed TOM/TIM nomenclature, the SGD nomenclature, or other specific naming. In this review, we will describe different names in parallel, which may be the most useful for readers when they read published literature.

outer membrane and Mia40/Tim401-Erv1 redox translocator in the IMS, have been identified, which has advanced our mechanistic insight into the mitochondrial protein trafficking significantly [2–5]. In this review, we would like to focus on mitochondrial protein trafficking at the outer mitochondrial membrane, which involves components in the cytosol, outer membrane, and IMS. Particular emphasis will be placed on the roles of chaperone functions of the translocator and related components mediating protein import and sorting.

2. Cytosolic system

More than 99% of mitochondrial proteins are encoded by the nuclear genome and synthesized in the cytosol. Most mitochondrial matrix proteins and some inner membrane proteins are synthesized as precursor proteins with an amino-terminal presequence, which contains a mitochondrial targeting signal and is cleaved off by the matrix processing peptidase in the matrix upon import [5,6]. Presequences are rich in positively charged residues and have the ability to form an amphiphilic helical structure [7–9]. There are some exceptional cases in which precursor proteins contain a presequence-like targeting signal in the middle [10] or at the C-terminus of the molecule [11]. On the other hand, polytopic inner membrane proteins, soluble IMS proteins, and outer membrane proteins are mainly synthesized without a cleavable presequence, but contain internal targeting signals within their mature parts [5]. Internal targeting signals of especially membrane proteins are often encoded as separate multiple signals and are associated with transmembrane (TM) segments.

As was suggested from the earlier electron microscopy observation that ribosomes are accumulated on the surface of yeast mitochondria [12], a subset of mitochondrial proteins are synthesized on mitochondrially bound ribosomes and are simultaneously transported into mitochondria. The nascent polypeptide-associated complex (NAC) in mammalian and yeast cells [13] and Mft52 [14] in yeast cells were found to deliver ribosome-associated nascent peptides to the mitochondrial surface for such co-translational protein import. Several mRNA-binding proteins were found to be responsible for targeting mRNA to the mitochondrial surface for co-translational protein import, as well [15]. The mechanism of co-translational import may increase targeting efficiency of mitochondrial proteins and/or facilitate translocation of otherwise tightly folded proteins across mitochondrial membranes [16–18]. Many other mitochondrial proteins are nonetheless synthesized on cytosolic ribosomes and imported into mitochondria in a posttranslational manner.

Mitochondrial precursor proteins tend to aggregate as they are often subunits of oligomeric protein complexes or integral membrane proteins with hydrophobic segments. Presequences themselves have properties to induce aggregation by interacting with unfolded mature domains [19]. This is the reason why cytosolic chaperones are often called for maintenance of import competence of mitochondrial proteins after their synthesis. Those chaperones include Hsp70 (and its partner J proteins), Hsp90, and mitochondrial import stimulation factor (MSF). Cytosolic Hsp70s (Ssa proteins in yeast) are the first cytosolic chaperones that were shown to facilitate protein import into mitochondria and the ER both in vivo and in vitro [20–22]. In addition to Hsp70, a cytosolic NEM (N-ethylmaleimide)-sensitive factor was suggested to promote targeting of otherwise import incompetent precursor proteins to mitochondria in vitro [22], yet its identity still remains elusive. MSF in the mammalian cytosol was identified as another NEM-sensitive factor that stimulates import of urea-denatured precursor proteins into isolated mitochondria in vitro [23]. Analyses of the in vitro import of mitochondrial precursor proteins preloaded onto MSF suggested that MSF binds to Tom70, a receptor subunit of the TOM40 complex, and then forwards substrate proteins to Tom20, another receptor subunit of the TOM40 complex, upon ATP hydrolysis [24,25]. On the other hand, Hsp70 was suggested to transfer substrate mitochondrial precursor proteins to Tom20 without hydrolysis of ATP [25]. This led to the idea that requirement of external ATP (outside mitochondria) for protein import into mitochondria reflects involvement of MSF for targeting to mitochondria [25,26]. However, a later study revealed that the previously adopted ATP depletion protocol had an experimental pitfall, and showed that ATP outside the mitochondria is required for maintenance of solubility of mitochondrial precursor proteins, mainly by Hsp70, but not for the release of mitochondrial proteins from chaperones after binding to e.g. Tom70 of the mitochondrial surface [27]. Besides, Tom70 was found to function as a direct docking site for Hsp70 (and Hsp90 in mammalian cells) [28], allowing mitochondrial proteins to bind to the mitochondrial surface either via both Tom70 and Tom20 (Hsp70-dependent binding) or directly via Tom20 alone (Hsp70-independent binding) (Fig. 1). These divergent results on cytosolic chaperones and their cognate docking sites have not been completely resolved. Lack of the analyses of the functions of MSF or Bmh1 and Bmh2 in yeast [29] in vivo has also hampered understanding of the physiological roles of MSF in mitochondrial protein targeting.

3. The TOM40 entry gate

3.1. Receptors

Most mitochondrial proteins enter mitochondria via the general entry gate, the TOM40 complex in the outer membrane. The TOM40 complex consists of the core complex made up by Tom40, Tom22, Tom5, Tom6, and Tom7, and peripherally associated receptors, Tom20 and Tom70 (and a minor component Tom71) (Fig. 1). Among those subunits, only Tom40, Tom22, and Tom7 are commonly found in eukaryotes, suggesting that they represent subunits of the putative ancestral TOM40 complex [30].

The TOM40 complex contains three receptor subunits, Tom20, Tom22, and Tom70 (and Tom71), which recognize mitochondrial-targeting signals. Tom20 is anchored to the outer membrane by its N-terminal TM segment and exposes the C-terminal receptor domain to the cytosol [31–33]. Although fungal and animal Tom20s take the N_{in}–C_{out} topology to be integrated in the outer membrane, plant Tom20 has the structure similar to rat Tom20, but with the reverse, N_{out}–C_{in} topology [34]. Tom20 is the major import receptor that preferentially recognizes the targeting signals of presequence-containing proteins. The NMR structure of the receptor core domain of rat Tom20 in a complex with a presequence peptide showed that Tom20 has a hydrophobic groove on the surface, to which hydrophobic side of the amphiphilic helix of the presequence binds [35]. Several different 15N-labeled mitochondrial presequence peptides were analyzed for their Tom20-binding elements by NMR [36]. Tom20-binding elements were found to occupy different positions, either near the N-terminus or at the C-terminus, in the presequence and exhibit a common pattern described as $\phi\chi\chi\phi\phi$ (ϕ is a hydrophobic amino acid, and χ is any amino acid). A recent analysis of the crystal structures of the Tom20-presequence complex further revealed that the bound peptide can take two different relative geometries to the hydrophobic groove, allowing the two key hydrophobic residues in the $\phi\chi\chi\phi\phi$ motif formed by diverse presequences to be best recognized by Tom20 without an induced fit mechanism [37].

In addition to the receptor function, the hydrophobic groove for presequence binding may potentially function as a chaperone to bind to unfolded polypeptides. Indeed, the recombinant cytosol domain of human Tom20 was found to prevent citrate synthase from heat-induced aggregate formation at high temperature [38]. After release of the presequence from Tom20 to downstream components of the TOM40 complex, the hydrophobic groove of Tom20 may perhaps shield the hydrophobic segment of the mature-domain polypeptide to reduce the risk of undesired interactions with other unfolded proteins

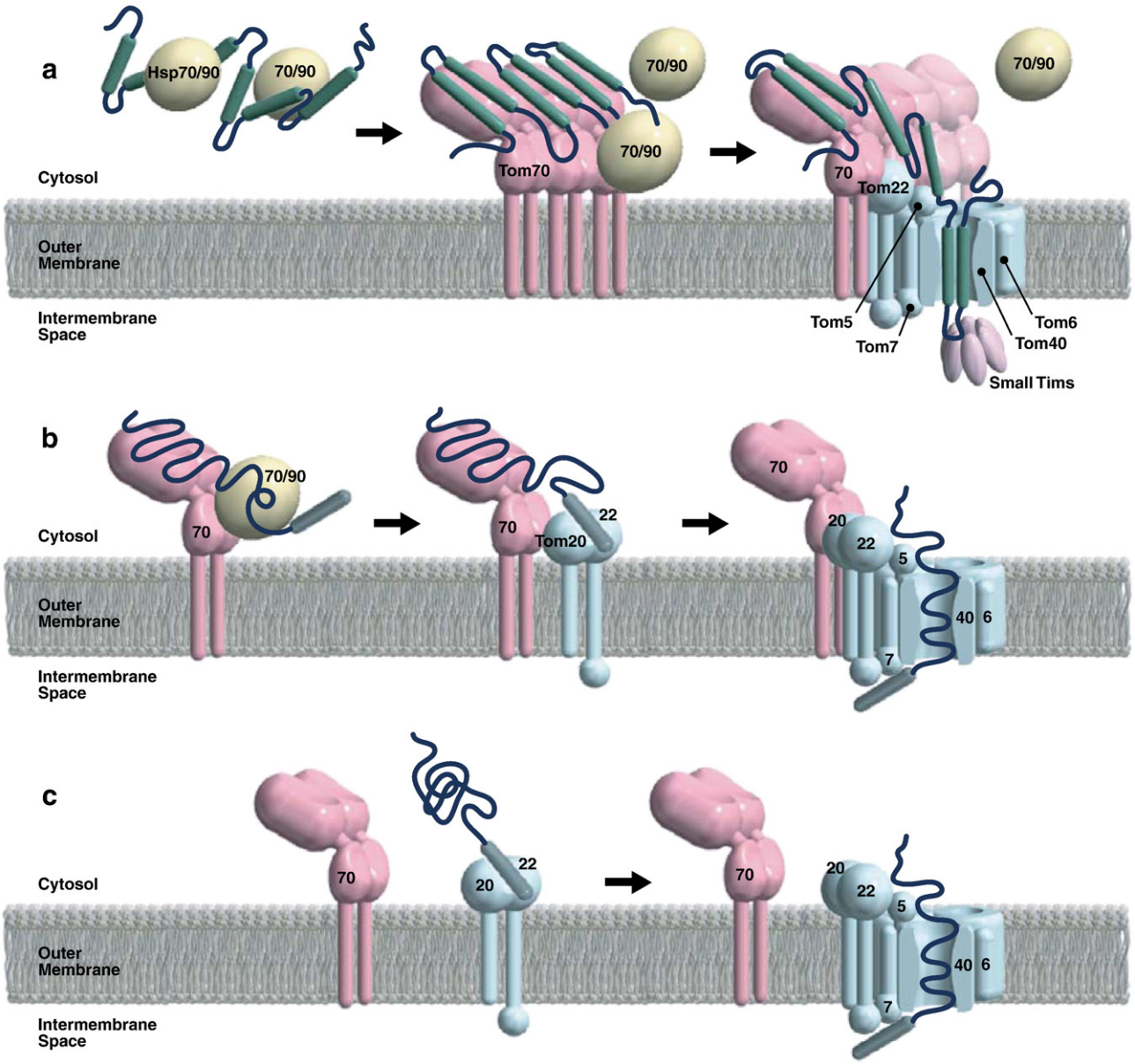

Fig. 1. Translocation of precursor proteins through the TOM40 complex. (a) A presequence-less, Tom70-dependent precursor protein. Many presequence-less precursor proteins including carrier proteins require Hsp70/Hsp90 for maintaining their import competence. Hsp70/Hsp90 docks onto Tom70 and the precursor proteins are transferred to Tom70, which may prevent precursor aggregation. Then the precursor proteins are transferred to the Tom40 channel likely via Tom22 and Tom5. After translocation through the TOM40 channel in a loop conformation, the precursor proteins bind to small Tim proteins in the IMS. (b) A presequence-containing, Tom70-dependent precursor protein. Some presequence-containing precursor proteins require cytosolic chaperones and dock onto Tom70. While Tom70 prevents aggregate formation of the precursor proteins, Tom20 and Tom22 recognize the targeting signal in the presequence. Then the precursor proteins are transferred to the TOM40 channel likely via Tom5. The inner wall of TOM40 channel may function as a chaperone for the unfolded mature part of the precursor proteins while the N-terminal presequence binds to the *trans* site of the TOM40 complex. (c) A presequence-containing, Tom70-independent precursor protein. Many presequence-containing proteins are directly recognized by Tom20 and Tom22 through their interactions with the presequence. Subsequent steps are similar to (b).

in the cytosol. However, physiological relevance of such a chaperone-like ability of Tom20 in vitro awaits further investigation.

Tom22 is anchored in the outer membrane by its hydrophobic TM segment in the middle of the molecule, exposing the N-terminal and C-terminal domains to the cytosol and IMS, respectively [39–41]. The cytosolic and IMS domains of Tom22 have been suggested to constitute distinct binding sites for presequences on both sides of the outer membrane, in cooperation with Tom20 on the cytosolic side (*cis* site) and with Tom40 and Tom7 on the IMS side (trans site), respectively [42–47]. The N-terminal cytosol domain of fungal and animal Tom22s consists of a highly acidic region containing many Glu/Asp residues followed by a more conserved, but neutral region. Sequence comparison shows that plant Tom22s apparently lack the acidic region, but still retain the neutral region [30]. The unusually high content of Glu/Asp residues in the acidic region of the cytosol domain of fungal and animal Tom22s suggests that it is likely 'natively unfolded'. However, irrespective of its folding state, the acidic region may well contribute to salt-sensitive binding of positively charged presequences. Recent analyses on the in vitro protein import into yeast mitochondria lacking one of the two receptor domains of Tom20 and Tom22 revealed surprising similarity for the substrate specificities between Tom20 and Tom22 [48]. This suggests that those two receptor subunits are involved in the same pathway of targeting signal recognition in import. Since the N-terminal region of the cytosolic domain of yeast Tom22 is rich in acidic residues, Tom20 and Tom22 may well recognize opposite sides of the amphiphilic helix of

the same presequence in the same step, Tom20 recognizing the hydrophobic surface and Tom22 the hydrophilic surface [48]. Nevertheless, the role of Tom22 as a *cis*-site receptor in mitochondrial targeting-signal recognition may not be crucial because replacement of 15 of 19 acidic residues in the cytosol domain of Neurospora Tom22 to neutral residues does not significantly affect mitochondrial protein import [49]. Like Tom20, the recombinant cytosol domain of human Tom22 was also shown to prevent citrate synthase from heat-induced aggregate formation [38].

Tom70 is anchored to the outer membrane in a dimeric form by its N-terminal TM segment and exposes the C-terminal receptor domain to the cytosol [50–52]. In contrast to Tom20, Tom70 preferentially recognizes presequence-less inner membrane proteins including carrier proteins (e.g. ADP/ATP carrier and phosphate carrier). Although a peptide-scan analysis showed that Tom70 binds to multiple segments throughout the polypeptide chain of non-cleavable phosphate carrier [53], little is known about the precise Tom70 recognition motifs in carrier proteins or other Tom70 substrates. The crystal structure of the cytosol domain of yeast Tom70 shows clustering of 11 TPR motifs into two sub-domains connected by a flexible linker [54]. The C-terminal sub-domain contains a large putative substrate precursor binding pocket, which consists of many hydrophobic residues in the interior and some charged residues at the opening (Fig. 1a). As described above, Tom70 not only functions as a receptor but also functions as a docking site for cytosolic chaperones such as Hsp70 (in yeast and mammals) and Hsp90 (mammals) to receive mitochondrial precursor proteins [28] (Fig. 1a). The N-terminal sub-domain of yeast Tom70 offers a peptide-binding groove for the C-terminal EEVD motif of Hsp70 and Hsp90, thereby contributing to docking of the Hsp70-substrate complex.

Tom71 is a homolog of yeast Tom70 with high sequence identity and is a minor subunit of the TOM40 complex [55,56]. Recently, the X-ray structure of the complex of Tom71 and Hsp70/Hsp90 derived C-terminal peptides containing the EEVD motif was determined [57]. Comparison of the determined 'open' structure of Tom71 with the previously determined 'closed' structure of Tom70 [54] suggests that binding of the Hsp70/Hsp90 C-terminal peptide to the N-terminal sub-domain of Tom71 or Tom70 will lead to significant opening of the precursor-binding pocket in the C-terminal sub-domain and bringing it closer to Hsp70/Hsp90 [57]. After dissociation of the chaperone-substrate complex, Tom70 likely takes over the role of maintaining solubility of the bound substrate protein from Hsp70/Hsp90, which may facilitate efficient substrate transfer to the downstream components such as Tom20, Tom22, and Tom40 of the TOM40 complex [58] (Fig. 1a and b). This hypothesis is supported by the observation that Tom70 itself has an ability to maintain solubility of aggregate-prone proteins in vitro [59]. Release of the loaded precursor proteins from Tom70 may again require ATP, but precise molecular basis of this ATP requirement still remains elusive [27]. Tom70 and Tom71 are also suggested to recruit Mfb1, a soluble F-box protein required for mitochondrial morphologenesis, to the mitochondrial membrane [60], although it is unclear whether they are specific receptors or chaperones for Mfb1.

3.2. Tom40 pore

Mitochondrial precursor proteins accepted by the receptor subunits are allowed to cross the outer membrane through the protein-conducting channel of the TOM40 complex, mainly made by Tom40. Tom5, one of the three small subunits of the TOM40 complex with a single transmembrane segment, functions downstream of Tom22, but upstream of Tom40 along the import pathway [61]. Tom5 has a small cytosolic domain with acidic residues, which likely guides positively charged presequences to the channel(s) of the TOM40 complex. The intact TOM40 complex appears to have two to three pores [62,63], although biological significance of the presence of multiple pores in a single TOM40 complex remains unclear. The multi-pore assembly of the TOM40 complex is stabilized by the two subunits, Tom22 [64] and Tom6, the latter of which may function in an antagonistic manner with Tom7 [65]. Recent EM analyses of the yeast TOM40 complex showed that the TOM40 complex has near-threefold symmetry with three globular ~50 Å domains with the Tom40 pore and Tom22 at the interdomain interfaces [66].

Presumably, Tom40 forms a β-barrel structure that functions as a protein-conducting channel. The size of the Tom40 pore is estimated to be 15–20 Å, which does not cause severe friction with translocating polypeptide chains, but can accommodate two transmembrane α-helices at the same time [67]. Since recombinant yeast Tom40 solubilized with detergent or reconstituted into lipid liposomes can bind to unfolded proteins and prevent them from aggregate formation, the inner wall of the Tom40 pore appears to possess some hydrophobic patches with an affinity for unfolded polypeptides [68]. *In vitro* import of mitochondrial proteins into isolated yeast mitochondria is blocked by addition of unfolded proteins, suggesting that Tom40 in intact mitochondria also have high affinity for unfolded proteins [68]. These findings suggest that Tom40 is not a passive pore but offers an optimized environment to translocating proteins through its affinity for non-native polypeptide segments (Fig. 1b and c). Preferential binding of the Tom40 pore to transiently unfolded segments of the precursor protein will shift the equilibrium to the unfolded state, thereby promoting at least partial unfolding of the substrate proteins, which is necessary for folded mature domains to thread the narrow translocation pore. Alternatively the Tom40 pore may accommodate, like molecular chaperones, the unfolded substrate protein in transit across the outer membrane until it is ready to be handed over to the downstream components in the IMS or inner membrane. Indeed, when translocation of the C-terminal part of the IMS-targeting precursor protein across the outer membrane is artificially blocked *in vitro*, a significantly large N-terminal part is accommodated in the chaperone-like TOM40 channel interior because of the lack of a strong pulling mechanism in the IMS [68].

Entry or confinement of the mature part of presequence-containing proteins into the narrow Tom40 pore should be entropically unfavorable. The current model suggests that this decrease in entropy is overcome by binding of the presequence to the *trans* site and that of the unfolded mature part to the inner wall of the Tom40 pore (Fig. 1b and c). The *trans* site of the TOM40 complex for presequence binding consists of the IMS domains/regions of Tom22, Tom7, and Tom40. Since binding of the positively charged presequence to the *trans* site of the TOM40 complex is mediated by electrostatic, not hydrophobic, interactions, the acidic IMS domain/region of Tom22 and Tom40 may primarily constitute the trans site while the basic IMS domain of Tom7 may assist proper interactions of the presequence with the *trans* site [46,47]. In contrast, binding of the unfolded mature domain to the Tom40 pore is primarily mediated by hydrophobic interactions [46]. Movement of the presequence-containing precursor through the Tom40 pore involves a shift of the presequence from the *cis* site to *trans* site and that of the unfolded mature part from Tom70 to the Tom40 pore interior. This transfer may well be driven by the affinity gradient of the components of the TOM40 complex properly aligned for interactions with the precursor protein.

Presequence-less carrier protein precursors consist of three separate modules, each consisting of a pair of TM segments connected by a matrix-exposed loop and containing distinct targeting information. In contrast to the presequence-containing precursors, carrier proteins may not enter the TOM40 channel as linear chains, but instead, thread the channel in a loop conformation with both termini remaining outside mitochondria while the middle part of the loop has reached the IMS to interact with small Tim proteins [69] (Fig. 1a). The three modules of carrier proteins bind to three dimers of Tom70 simultaneously, yet they are transferred to the TOM40 channel in a stepwise manner as the narrow TOM40 channel accommodates only a

single loop of polypeptides [69] (Fig. 1a). Entry of the loop structure containing a pair of TM segments into the Tom40 pore may be partly driven by binding of the hydrophobic TM segments to the pore interior through hydrophobic interactions. However, it is not clear if the *trans* site for presequence binding also provide binding site for the targeting signals in the loop structures of carrier proteins. Rather, small Tim proteins in the IMS may take the role of the *trans* site for presequences by trapping the loop structure from the IMS side to prevent its backsliding to the cytosol [69].

In order to complete the translocation through the TOM40 channel, the precursor protein needs to dissociate from the binding sites in the TOM40 complex. The presequence may well be transferred from the *trans* site of the TOM40 complex to the inner membrane receptor Tim50 [70,71]. This transfer is likely facilitated by transient, but direct interactions between Tom22 in the TOM40 complex and Tim23 and Tim50 in the TIM23 complex [70]. Clearance of the interior of the Tom40 pore for the release of the unfolded substrate polypeptide requires possible pulling mechanisms by the downstream transport machineries. Such mechanisms include the motor functions of the TIM23 complex with the MMC (mitochondrial Hsp70-associated motor and chaperone) or PAM (presequence translocase-associated motor) components driven by the membrane potential across the inner membrane and ATP hydrolysis in the matrix, and trapping by binding of small Tim proteins in the IMS or by tight folding upon introduction of disulfide bonds.

4. β-Barrel proteins

4.1. Initial steps for β-barrel protein assembly

Gram-negative bacteria are surrounded by two envelope membranes, and the outer envelope membrane contains >70 different monomeric or trimeric β-barrel membrane proteins [72]. Outer membrane β-barrel proteins are translocated across the inner membrane by the Sec machinery and traverse the periplasmic space with the aid of periplasm chaperones, Skp and SurA [73]. Then β-barrel proteins are integrated into the outer membrane with the aid of the β-barrel assembly machinery (the BAM complex), which was found to consist of Omp85 or BamA (YaeT) [74], and the other subunits (BamB, BamC, BamD, and BamE) [73,75]. In parallel with identification of the BAM pathway for the prokaryotic β-barrel membrane protein assembly, a new pathway was identified 6 years ago for the assembly of β-barrel membrane proteins in the eukaryotic mitochondrial outer membrane, as well [76] (Fig. 2). Four β-barrel membrane proteins, Tom40, porin (two isoforms), Sam50/Tob55, and Mdm10, have been identified so far in yeast mitochondria. Assembly of the *in vitro* synthesized Tom40 precursor into the TOM40 complex in isolated mitochondria proceeds through several distinct steps [77]. Tom40 is first recognized and translocated across the outer membrane by the TOM40 complex. Then Tom40 is received by the small Tim complexes, the Tim9–Tim10 or Tim8–Tim13 complex, in the IMS [78,79]. The Tim9–Tim10 and Tim8–Tim13 complexes are 70-kDa soluble hetero-hexamers containing three subunits of each protein. The Tim9–Tim10 complex facilitates transfer of the substrates for the TIM22 complexes while the Tim8–Tim13 complex plays a similar role, but for a limited number of substrates. The crystal structures of the human and yeast Tim9–Tim10 complexes show that they form α-propeller-like structures with alternating subunits [80,81]. Those unique tentacle-like hexamers may mask hydrophobic regions of substrate proteins, pointing to the important roles of small Tim proteins as chaperones in preventing hydrophobic transported proteins from aggregate formation in the IMS.

4.2. TOB/SAM complex

Guided by the small Tim proteins, Tom40 is assembled into the 250 kDa assembly intermediate with the second translocator in the outer membrane, the TOB or SAM complex. The TOB core complex consists of Sam50/Tob55 [82–84], Sam35/Tom38/Tob38 [85–87], and Sam37/Mas37/Tom37 [76,88]. Sam50/Tob55 is a β-barrel protein itself and a homolog of Omp85/BamA in the bacterial outer membrane. Sam50/Tob55 consists of the N-terminal POTRA domain in the IMS and the C-terminal pore forming β-barrel domain. The POTRA domain of Sam50/Tob55 may, like that of bacterial Omp85, function as a receptor for β-barrel proteins [89]. However deletion of the POTRA domain does not affect yeast cell growth or the import of β-barrel proteins, so that its precise role in β-barrel protein recognition requires further analyses [90]. On the other hand, the C-terminal β-barrel domain of Sam50/Tob55 may play a central role in formation of the β-barrel structure of the substrate proteins. Sam50/Tob55 forms a pore-like structure with a diameter of ~40–50 Å and a fivefold rotational symmetry and, after reconstitution into a lipid bilayer, exhibits a channel activity [82,90]. The interior of the β-barrel domain of Sam50/Tob55 may thus provide a scaffold for organization of the β-segments of substrate proteins into a barrel structure, or alternatively, multiple Sam50/Tob55 molecules in the TOB complex form a cavity, in which substrate proteins may form β-barrel structures.

Sam35/Tom38/Tob38 and Sam37/Mas37 are peripheral membrane proteins that are partially exposed to the cytosol. Sequence

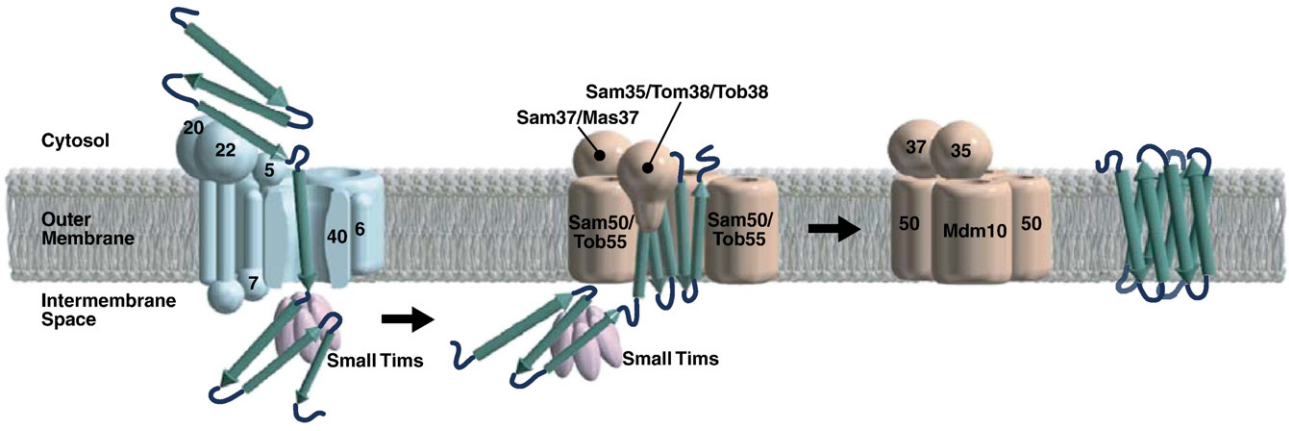

Fig. 2. Assembly pathway of β-barrel membrane proteins of the outer mitochondrial membrane. β-Barrel membrane proteins are recognized by Tom20 and Tom22, and move across the TOM40 channel to reach the IMS. In the IMS, the β-barrel membrane proteins bind to small Tim proteins, which prevent their aggregate formation. Then the β-barrel membrane proteins associate with the TOB complex without Mdm10, which promotes insertion of the β-barrel membrane proteins into the outer membrane. Tom40 requires Mdm10 in the TOB complex to dissociate from the TOB complex.

alignment of β-barrel proteins revealed the presence of a β-signal in mitochondrial, not bacterial, β-barrel proteins; β-signal is a short C-terminal sequence probably functioning as a sorting signal of mitochondrial β-barrel proteins [90]. Since recombinant Sam35/Tom38/Tob38 binds to β-signal peptides, Sam35/Tom38/Tob38 was proposed to act as a receptor of the β-signal [90]. However, partial exposure of Sam35/Tom38/Tob38 as a peripheral membrane protein to the cytosol and its receptor function in the IMS at the same time pose a topological problem for Sam35/Tom38/Tob38. Sam37/Mas37 was initially thought as a partner protein for the receptor Tom70 of the TOM40 complex, but it was later shown to be a subunit of the TOB complex, not the TOM40 complex [76]. Although the role of Sam37/Mas37 in β-barrel protein assembly remains vague, Sam37/Mas37 may contribute to the stability of the TOB complex [86] and/or promote release of substrate proteins from the TOB complex [91].

Recent studies showed that Mdm10 is also a subunit of the TOB complex, but at the same time, Mdm10 constitutes the MMM1 complex tethering the ER and mitochondria to function in lipid biosynthesis [92,93]. Enigmatically, while Mdm10 is a subunit of the TOB complex, Mdm10 was shown to facilitate assembly of Tom40 after its dissociation from the TOB complex [92]. However, by using a system in which the Mdm10 level in the TOB complex, not in the MMM1 complex, can be altered, decrease in the Mdm10 level was now found to result in accumulation of *in vitro* imported Tom40 (a β-barrel protein) at the level of the TOB complex. Since on the other hand, increase in the Mdm10 level inhibited association of not only Tom40 but also other β-barrel proteins with the TOB complex, Mdm10 and premature β-barrel proteins appear to be mutually exclusive on the TOB complex, but only Tom40, not the other β-barrel proteins, requires Mdm10 for exit from the TOB complex [94]. Mmm1 and Mdm12 were also recently suggested to facilitate the β-barrel protein assembly [95]. However, since Mmm1 and Mdm12 are, like Mdm10, subunits of the ER-mitochondria tethering complex, functional defects of Mmm1 and Mdm12 may cause indirect effects on β-barrel protein assembly, e.g. through aberrant lipid composition of mitochondria.

After release from the TOB complex, β-barrel protein substrates are inserted into the outer membrane in β-barrel structures. In contrast to Mdm10, porin, and Sam50/Tob55, Tom40 takes further distinct and ordered steps to assemble into the architecture of the final TOM40 complex. These later steps of the Tom40 assembly were proposed to require functions of Mdm10, Mim1/Tom13, and other subunits of the TOM40 complex [85,92,96].

5. α-Helical membrane proteins

5.1. N-terminally anchored proteins

Unlike β-barrel membrane proteins in the bacterial outer membrane, the β-barrel membrane protein Tom40 associates with the other subunits of the TOM40 complex that are all anchored in the outer membrane by a single α-helical TM segment. Topogenesis and assembly of those and other α-helical outer membrane proteins were only recently characterized. Several subunits of the TOM40 and TOB complexes as well as Mim1/Tom13 have been suggested to facilitate those processes.

A subset of mitochondrial outer membrane proteins including Tom20, Tom70, Tom71, and OM45 (with an unknown function) are anchored to the outer membrane by their N-terminal α-helical TM segment in the N_{in}–C_{out} orientation (N-anchored mitochondrial outer membrane proteins). Although those proteins resemble ER-targeted membrane proteins with the type I (N_{in}–C_{out}) signal-anchor sequence at the N-terminus, precise sorting to either mitochondria or the ER is

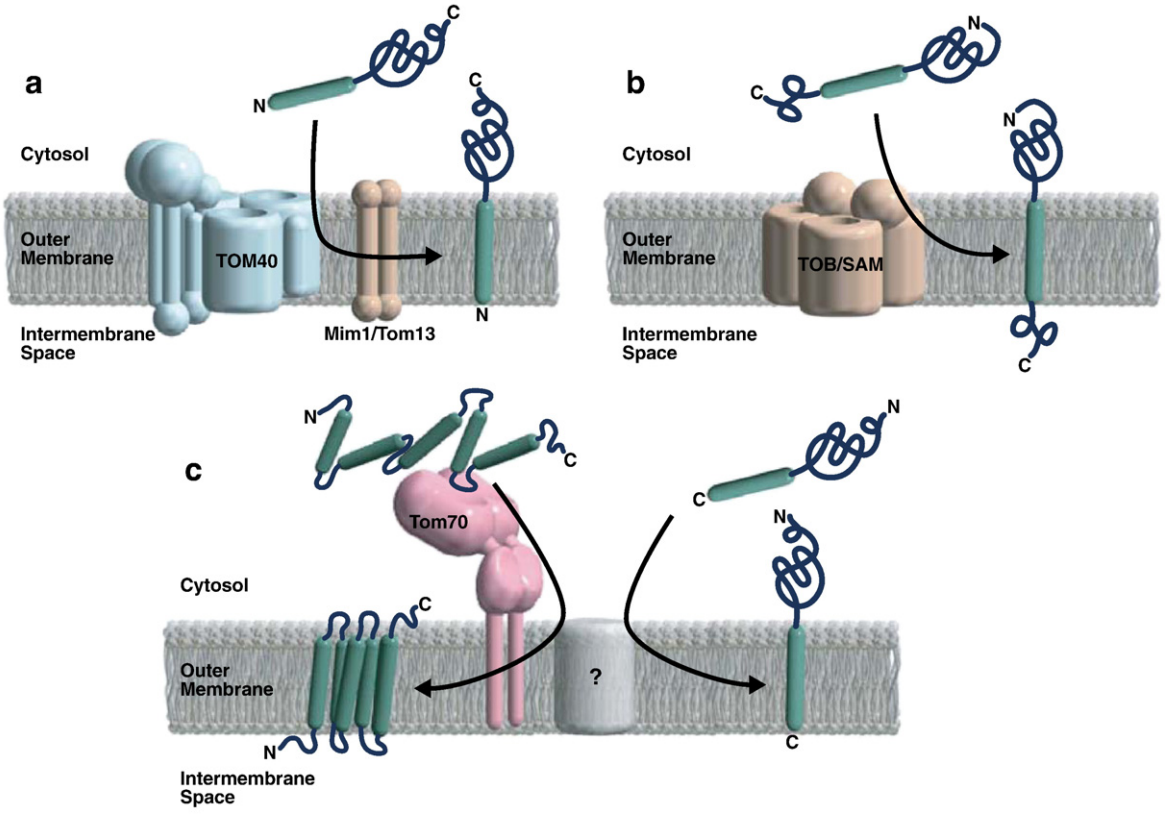

Fig. 3. Insertion pathways of α-helical membrane proteins into the outer mitochondrial membrane. (a) N-terminally anchored proteins. Many N-terminally anchored proteins require the TOM40 complex, but not its channel, to be inserted into the outer membrane. Some N-terminally anchored proteins may require Mim1/Tom13 for membrane insertion. (b) Tom22 with a TM segment in the middle of the molecule. Tom22 requires the TOB complex for its insertion into the outer membrane. (c) Polytopic outer membrane proteins and C-terminally anchored proteins. Mammalian polytopic outer membrane proteins may require Tom70, but not the other components of the TOM40 complex, for their insertion into the outer membrane. The polytopic outer membrane proteins may share at least partly the membrane insertion pathway with C-terminally anchored proteins.

achieved *in vivo*. In mammalian cells, N-terminal TM segment with a moderate hydrophobicity and a net positive charge in the C-terminal flanking region were found to function as the mitochondrial targeting signal, evading SRP-dependent ER targeting [97].

Assembly of N-anchored mitochondrial outer membrane proteins does not require receptor subunits such as Tom20 or Tom70 of the TOM40 complex, yet many of them still depend on the TOM40 complex for their membrane insertion. In bacterial inner membrane and eukaryotic ER membrane, the Sec (SecYE or Sec61) translocators contain a protein-conducting channel made up of bundles of α-helical TM segments [98]. The Sec machinery can undergo lateral opening without significant destabilization of the entire complex structure to release the α-helical TM segment(s) of substrate membrane proteins directly into the lipid bilayer. However, because the Tom40 channel is formed by the β-barrel structure, its opening would require energetically unfavorable disruption of many hydrogen bonds between the β-segments. This suggests that lateral release of α-helical TM segment from the Tom40 channel into the lipid bilayer may be unlikely. Instead, Tom20 was suggested not to use the Tom40 pore, but to be inserted into the outer membrane in the α-helical structure at the interface between the TOM40 core complex and the lipid phase of the membrane [99] (Fig. 3a). Recently, Mim1/Tom13 was found to affect assembly of not only Tom40 [85,100] but also Tom20; Mim1/Tom13 may facilitate insertion of Tom20 into the outer membrane [101] or docking to the TOM40 complex [102]. Broader roles of Mim1/Tom13 were further suggested for insertion of Tom20, Tom70, and Tom6 into the outer membrane as well as assembly of Tom5 and Tom7 into the TOM40 core complex [100].

5.2. C-terminally anchored proteins

The mitochondrial outer membrane contains several membrane proteins spanning the membrane with a single TM segment that is located away from the N-terminus. Tom5, Tom6, Tom7, and Tom22 of the TOM40 complex are anchored to the outer membrane by their TM segment at the C-terminus (Tom5, Tom6, and Tom7) or in the middle (Tom22) of the molecules in the N_{out}–C_{in} topology. Functional defects of Sam50/Tob55, Sam35/Tom38/Tob38, and Sam37/Mas37 of the TOB complex were found to, whether directly or indirectly, affect not only the assembly of Tom22 into the final TOM40 complex but also the efficiency of the membrane insertion of Tom22 [103] (Fig. 3b). Tom5 and Tom20 are required for proper assembly of Tom22 into the final TOM40 complex, as well [48,103]. On the other hand, defects of Sam37/Mas37, not Sam50/Tob55 or Sam35/Tom38/Tob38, apparently impair the assembly of Tom5, Tom6, and Tom7 into the TOM40 complex, but not the preceding step of their membrane insertion [103].

Both ER and mitochondrial membranes contain specific sets of C-terminally anchored or tail-anchored (TA) proteins. In contrast to the ER-targeted N-terminally anchored proteins, TA proteins inevitably require post-translational pathways for their insertion into the ER and mitochondrial membranes. Recently, a set of secretory-pathway TA proteins were found to associate with a cytosolic ATPase Get3 (yeast) or Asna1/TRC40 (mammal), which are delivered to the Get1/Get2 complex, receptors for Get3, in the ER membrane [104–106]. Get3 shuttles between the ER and cytosol to function as a TA-protein receptor as well as a chaperone, and in its absence, secretory-pathway TA proteins may insert into mitochondrial membranes. On the other hand, little is known for the factors involved in targeting of mitochondrial TA proteins to the mitochondrial outer membrane and subsequent membrane insertion (Fig. 3c). In mammalian cells, mitochondrial TA proteins appear to share a common targeting pathway independently of cytosolic factors or the TOM40 complex, although cytosolic chaperones may be required to keep TA proteins import competent [107]. In yeast, a mitochondrial TA protein, Fis1, was also found to be inserted into the mitochondrial outer membrane independently of the TOM40 or TOB components [108] (Fig. 3). At the moment, it is not clear if mitochondrial TA proteins require specific proteinaceous components other than appropriate lipid compositions in the mitochondrial outer membrane for their insertion.

5.3. Polytopic outer membrane proteins

The mitochondrial outer membrane contains several proteins that span the membrane multiple times. For example, yeast Fzo1p and its mammalian counterparts, Mfn1 and Mfn2, a mitochondrial ubiquitin ligase (MITOL), and the peripheral benzodiazepine receptor (PBR) are inserted into the mitochondrial outer membrane through two, four, and five TM segments, respectively. Integration of those polytopic membrane proteins into the human outer mitochondrial membrane depends on Tom70, but takes place independently of Tom20, Tom22, and Tom40 [109] (Fig. 3c). Nevertheless, those polytopic membrane proteins share at least partly the membrane insertion pathway with C-terminal TA proteins after release from Tom70, and importantly require components in the IMS for its membrane insertion [109]. Metaxin 1, a mammalian homolog of yeast Sam37/Mas37 was shown to facilitate insertion of PBT into the outer membrane [110].

6. Outlook

We have witnessed significant accumulation of knowledge on the functions of the TOM40 and TOB complexes in the outer membrane in recent years. However, we still anticipate discovery of more components and pathways for e.g. insertion of α-helical membrane proteins into the outer membrane and perhaps the inner membrane. Soluble factors functioning as a chaperone or targeting factor in the cytosol *in vivo* may also await identification. Although high-resolution structures have been revealed for several chaperones in the cytosol and IMS and for receptor domains of the TOM40 complex, entire structures of the membrane-embedded translocator complexes have escaped determination at atomic-resolution. Evidently, more structural information as well as that on dynamic interactions between translocator components and/or soluble factors will be essential for precise understanding of the mechanisms of mitochondrial protein transport. Finally, structural and functional maintenance of multi-subunit membrane protein complexes at the mitochondrial outer membrane is an important subject of future studies in close relation to mitochondrial biogenesis.

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